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Original Research Article

Vitamin A injection at birth improves muscle growth in lambs

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ABSTRACT

Vitamin A and its metabolite, retinoic acid (RA) play important roles in regulating skeletal muscle development. This study was conducted to investigate the effects of early intramuscular vitamin A injection on the muscle growth of lambs. A total of 16 newborn lambs were given weekly intramuscular injections of corn oil (control group, $n = 8$) or 7,500 IU vitamin A palmitate (vitamin A group, $n = 8$) from birth to 3 wk of age (4 shots in total). At 3 wk of age and weaning, biceps femoris muscle samples were taken to analyze the effects of vitamin A on the myogenic capacity of skeletal muscle cells. All lambs were slaughtered at 8 months of age. The results suggest that vitamin A treatment accelerated the growth rate of lambs and increased the loin eye area ($P < 0.05$). Consistently, vitamin A increased the diameter of myofibers in longissimus thoracis muscle ($P < 0.01$) and increased the final body weight of lambs ($P < 0.05$). Vitamin A injection did not change the protein kinase B/mammalian target of rapamycin and myostatin signaling ($P > 0.05$). Moreover, vitamin A upregulated the expression of *PAX7* ($P < 0.05$) and the myogenic marker genes including *MYOD* and *MYOG* ($P < 0.01$). The skeletal muscle-derived mononuclear cells from vitamin A-treated lambs showed higher expression of myogenic genes ($P < 0.05$) and formed more myotubes ($P < 0.01$) when myogenic differentiation was induced in vitro. In addition, in vitro analysis showed that RA promoted myogenic differentiation of the skeletal muscle-derived mononuclear cells in the first 3 d ($P < 0.05$) but not at the later stage ($P > 0.05$) as evidenced by myogenic gene expression and fusion index. Taken together, neonatal intramuscular vitamin A injection promotes lamb muscle growth by promoting the myogenic potential of satellite cells.

1. Introduction

Lamb meat production is increasing all over the world to meet the demands of increasing meat consumption (Ponnampalam et al., 2016). Muscle growth is achieved by the formation of muscle fibers in the embryo (Yan et al., 2013) and postnatal muscle fiber hypertrophy (Ontell et al., 1984). It is widely accepted that the majority of muscle fibers are formed before birth and the postnatal growth of muscle is through protein synthesis accompanied by the fusion of satellite cells into existing muscle fibers (Ontell et al., 1984; White et al., 2010; Yan et al., 2013; Zhu et al., 2004). Nutritional status at the neonatal stage greatly affects satellite cell proliferation which has long-term effects on muscle growth (MacGhee et al., 2017).

Vitamin A (also known as retinol) is an essential nutrient for animals. As the most active metabolite of vitamin A, retinoic acid (RA) is involved in the embryonic development of many tissues and organs, for instance, adipogenesis (Schwarz et al., 1997), myogenesis (Ryan et al., 2012) and neurogenesis (Yu et al., 2012). At the embryonic stage, RA promotes the development of the limb bud by recruiting muscle progenitor cells into the limb (S. Reijntjes et al., 2010). The classical way that RA regulates gene transcription is by

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acting as a ligand for RA receptors (RARs). RARs form a heterodimer with retinoid X receptors (RXR), and RA ligation of RAR changes the transcription factors that bind to the RAR-RXR heterodimer, which in turn changes the transcription pattern of the target genes (Chawla et al., 2001; Rochette-Egly and Germain, 2009). Retinoic acidpromotes myogenic differentiation of mouse myoblast C2C12 cells by activation of RAR and RXR signaling (Zhu et al., 2009). Many signaling pathways have been identified to be involved in RA promotion of myogenic differentiation. For instance, RA activates myogenesis through FGF8 signaling (Hamade et al., 2006), and also by antagonizing transforming growth factor-beta signaling via C/EBPβ (Lamarche et al., 2015). In domestic animals, supplementation of vitamin A to finishing cattle increases the growth rate but reduces the marbling index (Wang et al., 2007). Intramuscular injection of vitamin A at the neonatal stage enhances postnatal muscle growth by promoting myogenesis and increasing satellite cell density (Harris et al., 2018; Wang et al., 2018). In addition to the promotion of myogenic differentiation, RA is also proven to induce the formation of oxidative muscle fibers by upregulating the expression of *PPARGC1A* (Wang et al., 2018) and *PPARδ* (Kim et al., 2018) in bovine satellite cells.

Considering the strong promoting effects that neonatal vitamin A administration showed on muscle growth in cattle (Harris et al., 2018; Wang et al., 2018), it could be a simple but effective nutritional strategy to improve animal growth. Thus, in the current study, we explored the effects of intramuscular vitamin A injection at the neonatal stage on the muscle growth of lambs.

2. Materials and methods

2.1. Animal ethics statement

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanxi Agricultural University (sxnd202028).

2.2. Animal treatments and diets

A total of 80 purebred Hu sheep in similar physical conditions were randomly selected. All ewes had been pregnant twice previously (third pregnancy for the current experiment). All ewes were synchronized and inseminated with semen from one Dorper ram. Three pregnant ewes per stall were provided with free access to feed and water. The diet was formulated to meet National Research Council (2007) nutrient requirements for ewes. The number of fetuses was determined at 35 d of gestation using an ultrasound monitor. Only ewes with 2 fetuses were used for further experiments. At birth, one lamb from each pair of male twins was selected. A total of 16 lambs (weighing 3.5 ± 0.5 kg) were randomly assigned to 2 groups.

On the 2nd day of birth, lambs were injected with 7,500 IU vitamin A palmitate (product no. PHR1235, Sigma, Milwaukee, US) with corn oil as solvent (product no. C8267, Sigma, Milwaukee, US) or an equivalent volume of corn oil (control group) into the biceps femoris muscle. The lambs were injected once a week at a fixed time point for 3 wk and managed in pairs with ewes. The temperature of the sheep house was kept at about 10 °C and was well ventilated. On the 10th day of birth, the lambs were vaccinated against combined ovine/caprine braxy, struck, lamb dysentery and enterotoxaemia (Harbin Pharmaceutical Group Bio-vaccine Co., Ltd, Harbin, China), and then vaccinated against sheep pox (Harbin Pharmaceutical Group Bio-vaccine Co., Ltd, Harbin, China), Peste des petits ruminants (Tecon Biological Co., Ltd, Xinjiang, China) and foot-and-mouth disease (Inner Mongolia Bigvet Biotech Co., Ltd, Inner Mongolia, China) on d 28, 47 and 49, respectively. All lambs were weaned at the age of 12 wk and then transferred to finishing houses for further feeding. After weaning, lambs were fed ad libitum with a backgrounding diet for 55 d then transitioned to the finishing diet and had free access to clean water and a salt block. In addition, grass hay (peanut seedlings) for lamb growth was added during the finishing period. From weaning to harvest, the concentrate feed of the finishing diet increased from 0.3 to 1.75 kg (nutrient composition of the concentrate feed as shown in Table 1) and the grass hay increased from 0.25 to 0.5 kg per lamb per day (nutrient composition of the grass hay as shown in Table 2). The concentrate feed is a commercial diet (Shanxi Guannong Science and Technology Co., Ltd, China). For grass hay, the content of DM was determined by AOAC method (AOAC, 2000), and the contents of NDF and ADF were determined by Van Soest method (Van Soest et al., 1991). Crude protein and crude fat content were measured by the Kjeldahl and Soxhlet extraction methods, respectively (Li et al., 2022). The total ash content was determined by charring the samples in a crucible at a constant temperature of 600 °C for 40 min. All animals were weighed every week before weaning and once a month after weaning to record the growth rate of lambs in different groups. The feed intake and refusal of lambs were recorded every day and all lambs were slaughtered and sampled at the age of 8 months.

2.3. Collection and analysis of muscle biopsy

Lambs were surgically sampled in the 3rd and 12th wk. A small area of the biceps femoris of lambs in the right hind leg was scrubbed with iodophor. Besides, normal saline, 75% alcohol, epinephrine and surgical instruments were prepared for the subsequent sampling. Lambs were injected with 2% lidocaine and completely anesthetized, an incision of about 3 cm cut with a scalpel then about 2 g of skeletal muscle tissue was collected. The skin was then stitched up and the wound cleaned. Biopsy samples were divided into 4 parts: (1) the sample was placed in paraformaldehyde and embedded in paraffin to analyze the structure of muscle fiber; (2) the sample was ground with liquid nitrogen for

Table 1
Ingredients and nutrient levels of the concentrate feed at backgrounding and finishing stages of lambs (%, DM basis).

Ingredients	Backgrounding	Finishing
Corn	60	75
Wheat bran	11	0
Soybean meal	15	8
Soya bean cake	8	10
Premix (4%)[1]	5	5
Baking soda	1	2
Total	100	100
Nutrient level		
DM	87.4	87.3
CP	16.72	13.64
RUP	40.3	44.1
NEg, Mcal/kg	1.3	1.32
TDN	78.4	79
NDF	16.6	13.9
ADF	6.1	5.1
EE	3.8	3.9
Ca	1.14	1.12
P	0.49	0.43
Ca:P	2.32	2.6

DM = dry matter; CP = crude protein; RUP = rumen undegraded protein; NEg = net energy of growth; TDN = total digestible nutrients; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract.

[1] Premix (4%) contains vitamin A, vitamin D_3, vitamin E, $FeSO_4$, $Cu_2(OH)_3Cl$, $ZnSO_4$, $MnSO_4$, $Ca(IO_3)_2$, Na_2SeO_3, $CoCl_2$, Na_2SO_4, NH_4Cl, flavoring agent includes neotame and saccharin sodium (Purchased from Beijing Yinghuier Biotechnology Co., Ltd).

Table 2
Composition of the grass hay (%, DM basis).

Nutrient	Amount
DM	87.74
CP	12.32
EE	2.30
Ash	11.26
NDF	63.47
ADF	40.01

DM = dry matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber.

RNA extraction (q-RT-PCR) and Western blotting; (3) the sample was put into cold phosphate buffer saline (PBS) for satellite cell separation; (4) the sample was processed for cryosection. The skeletal muscle samples of the slaughtered lambs were treated with the above methods.

2.4. Hematoxylin-Eosin (H&E) staining and diameter measurement of myofibers

The trimmed skeletal muscle samples were fixed in paraformaldehyde, then successively dehydrated in ethanol and xylene, and finally embedded in paraffin. The embedded samples were cut to 5 μm thickness using a microtome (Leica, German), and sections were dewaxed and rehydrated by xylene and gradient ethanol. After that, H&E staining was used for histological examination. Finally, the myofiber diameter was measured using Image J software (Bethesda, MD, US) after images were taken by a microscope (DMi8, Leica, Germany). All samples were cut every 50 μm, at least 200 muscle fibers were counted in each visual field, and at least 3 repeats in each sample.

2.5. Immunohistochemical staining

Muscle tissues were frozen in liquid nitrogen, embedded in optimal cutting temperature and cut into 8 μm sections by a frozen cryo-microtome (Leica CM1950, Germany). Tissue sections were blocked with 2% goat serum in tris buffered saline containing 10% Triton X-100 and 1% bovine serum albumin (BSA) for 1 h, incubated with anti-PAX7 antibody (RRID: AB_528,428, DSHB, Iowa City, US) overnight at 4 °C and the corresponding fluorescent secondary antibody for 1 h at room temperature. Then, sections were mounted with 4',6-diamidino-2-phenylindole (DAPI) (Vector Lab, Burlingame, CA), and images were visualized under a DMi8 microscope (Leica, Germany).

2.6. Sheep primary myoblast isolation

Biopsied muscle tissues were washed sequentially through precooled 75% alcohol and PBS 3 times, the surrounding non-muscle tissue was removed, then the samples were cut into small pieces and 1 mg/mL type I collagenase added, and finally digested for 45 min in a 37 °C shaker at 100 rpm. The digested muscle mixture was centrifuged at 500 × *g* for 5 min, and then the supernatant was discarded. The sediment was resuspended and passed through 100 and 40 μm cell strainers in sequence. The filtrates were centrifuged (500 × *g*, 5 min) and cell pellets were resuspended with Dulbecco's modified eagle medium (DMEM), which contained 20% fetal bovine serum, 0.1% penicillin, and 0.1% streptomycin. Finally, the cells were seeded onto a cell culture dish precoated with 10% matrigel.

2.7. Culturing, differentiation, and immunocytochemical staining of sheep primary myoblasts

Primary cells were purified and cultured for 2 d to reach 100% confluence, then replaced with a differentiation medium containing 2% horse serum to induce myotube formation. After 3 and 6 d of differentiation, cells were fixed in cold 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 and blocked with 1% BSA. The cells were incubated with anti-myosin heavy chain (MHC) (RRID: AB2147781, DSHB, Iowa City, US) antibody at 4 °C overnight, followed by incubation with corresponding fluorescent secondary antibody (anti-mouse, no. 4408, Cell Signaling, Danvers, MA, US) for 1 h at room temperature. Finally, cells were mounted with DAPI (Vector Lab, Burlingame, CA), and images were visualized under a DMi8 microscope (Leica, Germany).

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from skeletal muscle samples or cultured cells using Trizol reagent (Sigma, Saint Louis, MO) and cDNA was synthesized by a reverse transcription kit (TAKARA Co., Ltd, Dalian, China). Q-RT-PCR was carried out using a CFX RT-PCR detection system (Bio-Rad, Hercules, CA) and SYBR Green RT-PCR kit (TAKARA Co., Ltd). The cycle parameters were as follows: 95 °C for 10 min; 45 two-step cycles of 95 °C for 15 s and 60 °C for 30 s. There were at least 3 repeats in each group. All primer sequences are listed in Table 3. The relative content of mRNA was standardized to the internal reference β-actin, and the relative changes in gene expression by the $2^{-\triangle\triangle Ct}$ method.

2.9. Western blotting

Protein in skeletal muscle was extracted with the RIPA lysis buffer (1% NaF (product no. 201154, Sigma, Milwaukee, US), 1% Na_3VO_4 (product no. 450243, Sigma, Milwaukee, US), 1% phenylmethylsulfonyl fluoride (PMSF, no. P0100, Solarbio, Beijing, China), 2% β-mercaptoethanol (product no. M6250, Sigma, Milwaukee, US), 0.1% protease inhibitor (no. A8260, Solarbio, Beijing, China), 1 × loading buffer at 10 mL constant volume). The extracted protein was separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; room temperature, 80 V for 0.5 h, 120 V for 1.5 h), and then transferred to a nitrocellulose membrane (4 °C, 100 V for 2 h). The nitrocellulose membrane was blocked in 5% skim milk powder (Sangon Biotech Co., Ltd, Shanghai, China) for 1 h, and then incubated with the primary antibody (4 °C, overnight) and the corresponding secondary antibody (room temperature, 1 h). Western blotting analysis was carried out with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, US), and the band density was normalized to β-tubulin content.

Table 3
Primer sequences for Real-time PCR.

Genes	Sequence (5'→3')	Product size, bp
PAX7	F: CGGGCATGTTTAGCTGGGAGA	107
	R: TCTGAGCACTCGGCTAATCGAAC	
MYF5	F: CCCACCAGCCCCACCTCAAGT	93
	R: GTAGACGCTGTCAAAACTGCTGCT	
MYOD	F: GAACTGCTACGACCGCACTTACT	111
	R: GAGATGCGCTCCACGATGCT	
MYOG	F: CTCAACCAGGAGGAGCGCGAC	131
	R: TTGGGGCCAAACTCCAGTGCG	
β-actin	F: CGGCTTTCGGTTGAGCTGAC	159
	R: GCCGTACCCACCAGAGTGAA	

PAX7 = paired box gene 7; *MYF5* = myogenic factor 5; *MYOD* = myogenic differentiation 1; *MYOG* = myogenin.

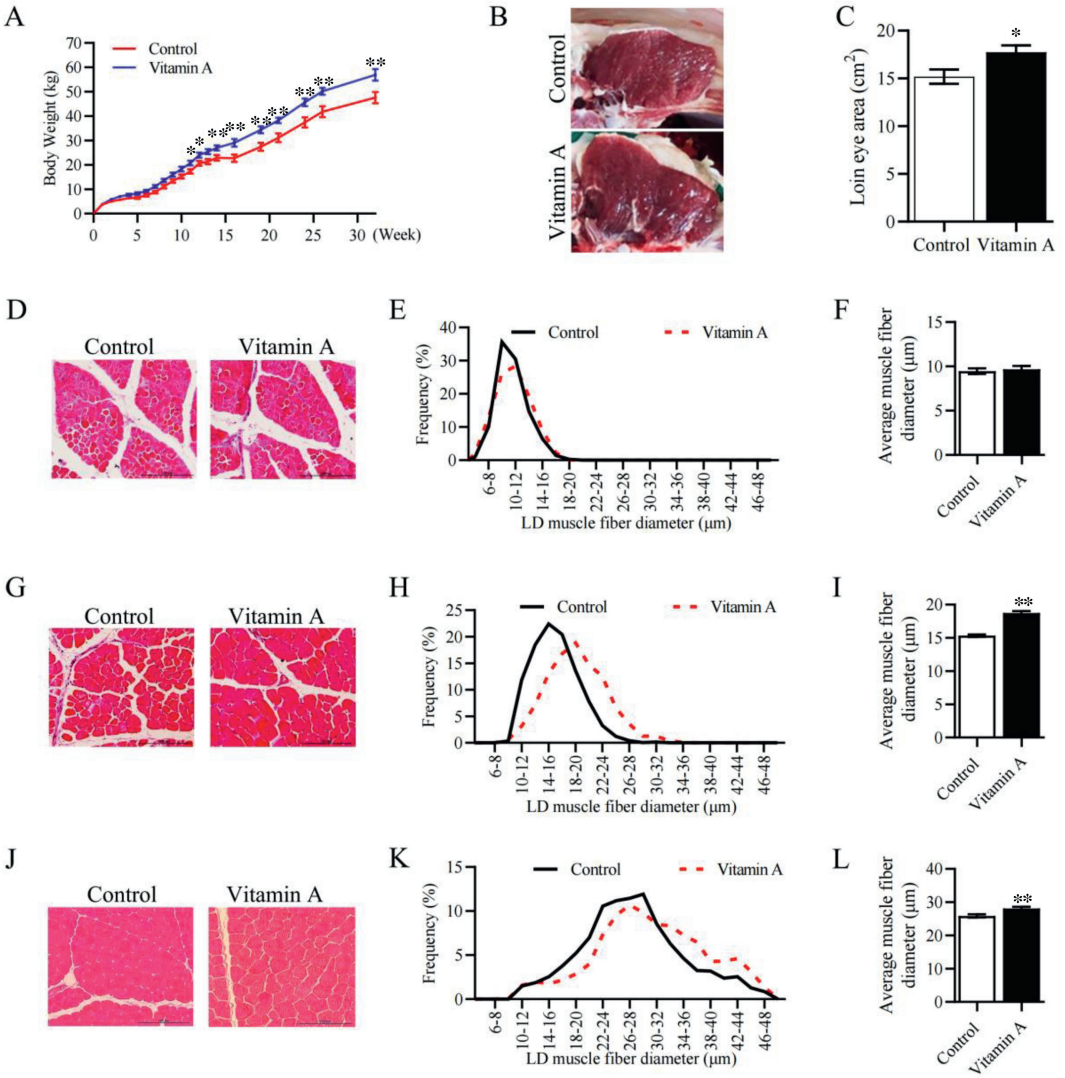

Fig. 1. Vitamin A injection promoted muscle growth of lambs. (A) Growth rate. (B) Representative image of loin eye muscle at harvest. (C) Average loin eye area at harvest. (D) H&E stained longissimus thoracis (LT) muscle in 3-wk-old lambs. (E) Distribution of fiber diameter of LT muscle in 3-wk-old lambs. (F) Average LT muscle fiber diameter in 3-wk-old lambs. (G) H&E stained LT muscle in 12-wk-old lambs. (H) Distribution of fiber diameter of LT muscle in 12-wk-old lambs. (I) Average LT muscle fiber diameter in 12-wk-old lambs. (J) H&E stained LT muscle at harvest. (K) Distribution of fiber diameter of LT muscle at harvest. (L) Average LT muscle fiber diameter at harvest. (mean ± SEM; $n = 8$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$).

Antibodies against protein kinase B (AKT, no. 92728), phospho-AKT (Ser473, no. 4060S), mammalian target of rapamycin (mTOR, no. 2972), phospho-mTOR (Ser2448, no. 2971), 4EBP1 (no. 9644), phospho-4EBP1 (Thr37/46, no. 2855), p70 S6 Kinase (no. 2708) and phospho-p70 S6 Kinase (Thr389, no. 9206), Smad2/3 (no. 3102 s) and phosphor-Smad3 (no. 9520 s) were purchased from Cell Signaling (Danvers, MA, US). Myostatin (MSTN) (sc-134,345) and MYOD (sc-760) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, US). Myosin heavy chain 2 (bs-10903R), MYOG (bs-3550R), and β-tubulin (bsm-33034M) were purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Goat anti-rabbit secondary antibody (926–32,211) and anti-mouse secondary antibody (926–68,070) were from LI-COR Biosciences (Lincoln, NE, US).

2.10. Statistical analysis

The data obtained in this experiment were processed using GraphPad Prism 9 software (Monrovia, CA, US). All data were analyzed using unpaired Student's *t*-test. Significance was accepted at $P < 0.05$. All data are expressed as means ± standard errors of the mean (SEM).

3. Results

3.1. Effects of vitamin A injection on growth performance in lambs

Intramuscular vitamin A injection at the neonatal stage significantly increased the growth rate of lambs (Fig. 1A, $P < 0.01$) and

Table 4
Effect of vitamin A injection on growth performance of lambs ($n = 8$).

Items	Control	Vitamin A	SEM	*P*-value
Initial BW, kg	3.76	3.85	0.201	0.67
Final BW, kg	47.56[b]	56.93[a]	3.347	0.01
ADG, kg/d	0.18[b]	0.22[a]	0.014	0.02
DMI, kg/d	1.75	1.78	0.112	0.76

BW = body weight; ADG = average daily gain; DMI = dry matter intake.
[a, b]Different superscripts in each row for each factor differ significantly ($P < 0.05$).

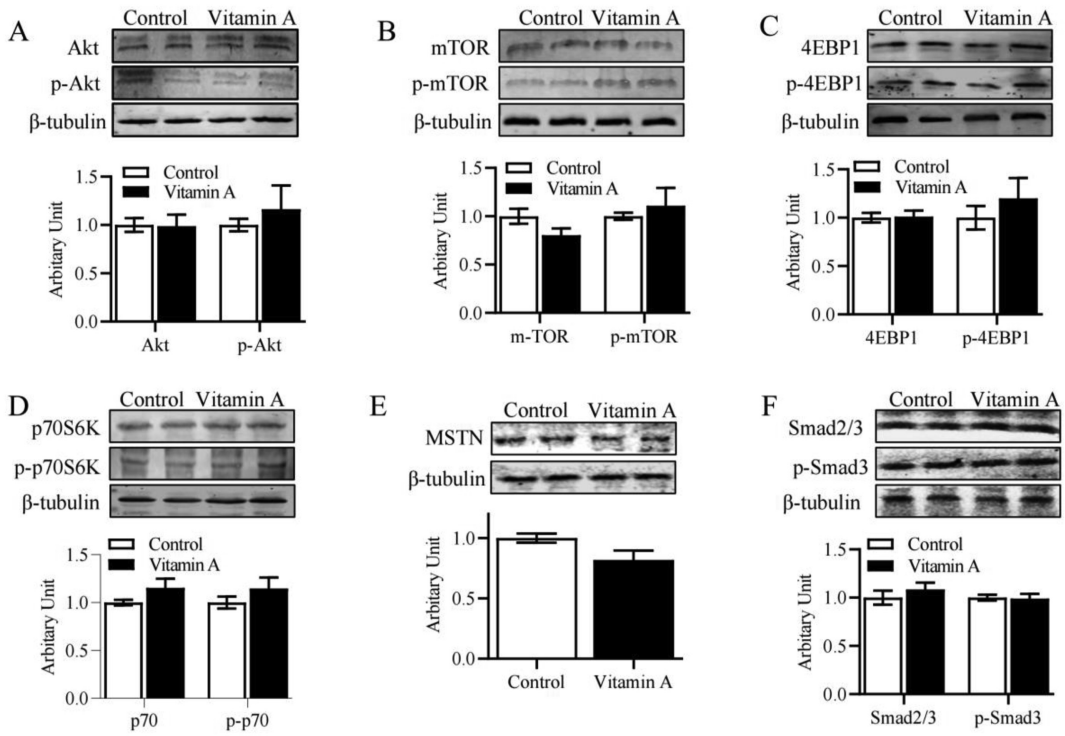

Fig. 2. Effects of vitamin A injection on Akt/mTOR signaling activity and MSTN content in longissimus thoracis muscle. (A) Total Akt and phosphor-Akt protein abundance in longissimus thoracis muscle. (B) Total mTOR and phosphor-mTOR protein abundance. (C) Total 4EBP1 and phosphor-4EBP1 protein abundance. (D) Total p70S6K and phosphor-p70S6K protein abundance. (E) Myostatin (MSTN) protein abundance. (F) Both Smad2/3 and phosphor-Smad3 protein abundance (mean ± SEM; $n = 8$ in each group).

increased the loin eye area of lambs at 8 months of age (Fig. 1B and C, $P < 0.05$), with no changes in DMI (Table 4, $P = 0.76$). No difference was found in the longissimus thoracis (LT) muscle fiber size at 3 wk of age (Fig. 1D–F, $P > 0.05$), however, vitamin A increased the LT muscle fiber size at 12 wk (Fig. 1G–I, $P < 0.01$) and 8 months of ages (Fig. 1J-L, $P < 0.01$).

3.2. The effects of vitamin A injection on Akt/mTOR signaling activity and myostatin content in LT muscle

We first determined whether vitamin A injection promotes muscle growth by activating signaling pathways involved in muscle protein synthesis. There was no difference in the protein content of AKT, phosphorylated AKT (Fig. 2A, $P > 0.05$), mTOR, phosphorylated mTOR (Fig. 2B, $P > 0.05$), 4EBP1, phosphorylated 4EBP1 (Fig. 2C, $P > 0.05$), p70S6K4, or phosphorylated p70S6K4 (Fig. 2D, $P > 0.05$) in LT muscle between the control and vitamin A treated lambs. In addition to AKT signaling, we further analyzed the content of MSTN, a myokine that inhibits muscle growth (Langley et al., 2002), and no difference was detected between the control and vitamin A-treated lambs (Fig. 2E, $P > 0.05$). Consistently, total Smad2/3 and phosphorylated Smad3 protein abundance were not altered between groups (Fig. 2F, $P > 0.05$). The results suggested that vitamin A injection at the neonatal stage did not change signaling involved in muscle protein synthesis.

3.3. Vitamin A injection increased the number of satellite cells and promoted myogenesis

We then explored the effects of vitamin A injection on the myogenic potential of cells residing in the skeletal muscle of lambs. Vitamin A treated animals had more $PAX7^+$ satellite cells in the skeletal muscle but the difference was not statistically significant (Fig. 3A and B, $P > 0.05$). In addition, higher expression of *PAX7* (Fig. 3C, $P < 0.05$) was detected in the LT muscle of 3-wk-old lambs. At 3 wk of age, no difference in the expression of myogenic genes including *MYF5*, *MYOD*, and *MYOG* was detected (Fig. 3D, $P > 0.05$). However, at 12 wk of age, vitamin A-treated lambs had higher expressions of *MYOD* and *MYOG* in the LT muscle (Fig. 3E, $P < 0.01$). There was no difference in protein levels of MYOD (Fig. 3F, $P > 0.05$) but MYOG was upregulated by vitamin A (Fig. 3G, $P < 0.05$). When compared with the control, mononuclear cells derived from the LT muscle of vitamin A-treated lambs had higher expression of *MYF5*, *MYOD,* and *MYOG* and formed more myotubes after 6 d of myogenic differentiation (Fig. 3H–J, $P < 0.05$). Consistently, higher MHC protein content was observed in the cells derived from the LT muscle of vitamin A-treated lambs (Fig. 3K, $P < 0.01$). These data indicated that vitamin A injection at the neonatal stage improved the myogenic potential of skeletal muscle.

3.4. RA promoted early differentiation of sheep primary myoblasts

We further investigated the effects of RA, the most active metabolite of vitamin A, on the myogenic differentiation of primary mononuclear cells at different stages. The cells were treated with all-trans RA at 0 to 3 d, 4 to 6 d, or 0 to 6 d. Cells treated with RA for the whole 6 d differentiation period (0 to 6 d) or during 0 to 3 d formed more myotubes and had higher fusion indices (Fig. 4A and B, $P < 0.05$), however, cells treated with RA during the last 3 d of myogenesis did not affect the formation of myotube or the fusion index (Fig. 4A and B, $P > 0.05$). Consistently, cells treated with RA for 0 to 6 d (Fig. 4C and F, $P < 0.05$) and 0 to 3 d (Fig. 4D and G, $P < 0.05$) had higher expression of *MYOD* and *MYOG* mRNA and higher expression of MHC protein, but no difference in these genes or protein was detected when cells were treated with RA for 4 to 6 d (Fig. 4E and H, $P > 0.05$). These data proved that retinoic acid played

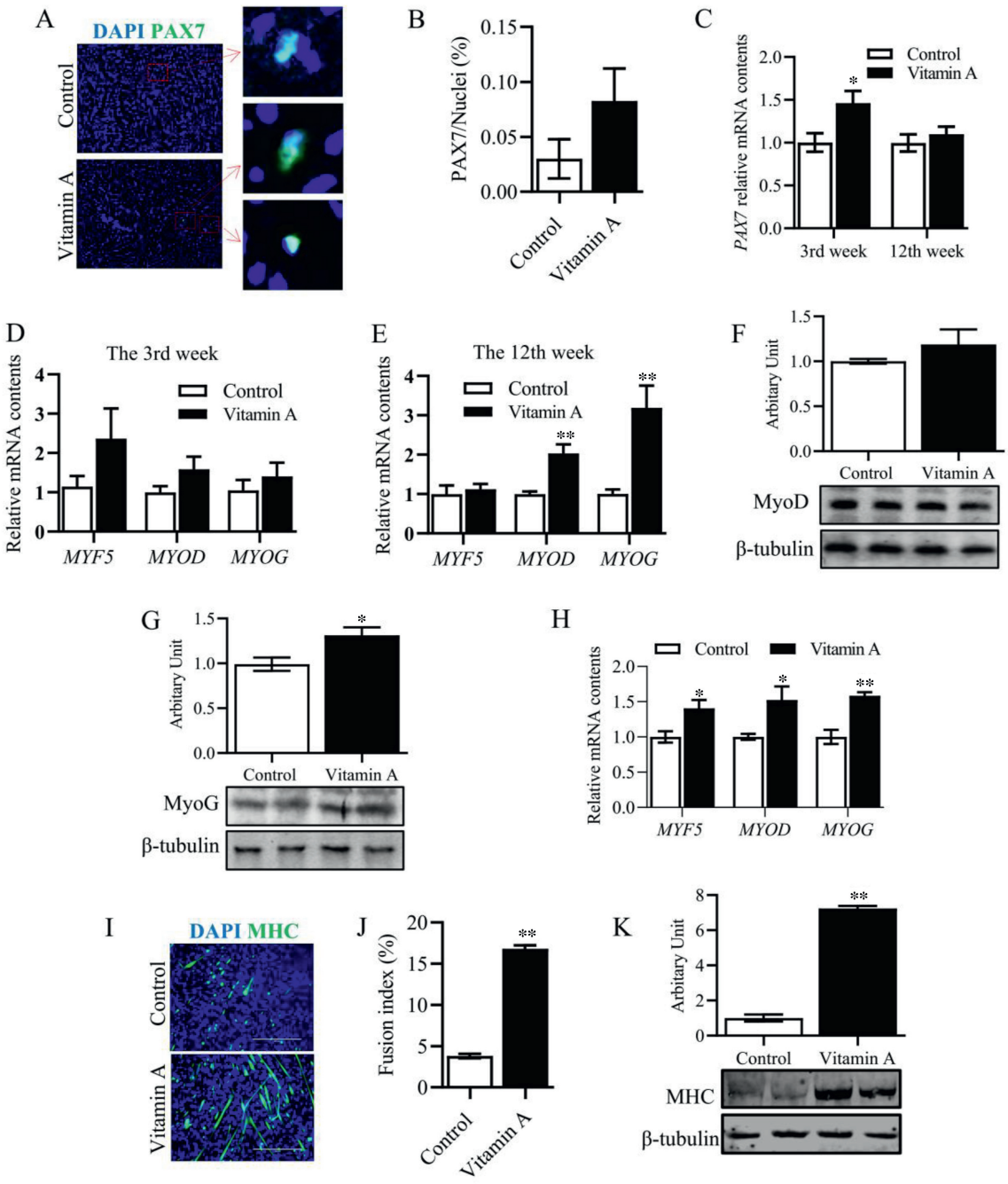

Fig. 3. Vitamin A injection increased the number of satellite cells and upregulated the expression of myogenic genes. (A) Muscle biopsy stained by PAX7. (B) Quantification of PAX7$^+$ cells in muscle biopsy. (C) Relative mRNA of *PAX7* in lambs at 3 and 12 wk of age. (D) The level of myogenic mRNAs in 3-wk-old lambs. (E) The level of myogenic mRNAs in 12-wk-old lambs. (F) Protein level of MYOD in 12-wk-old lambs. (G) Protein level of MYOG in 12-wk-old lambs. (H) The level of myogenic mRNAs after 6 d of myoblast differentiation. (I) Myotubes were visualized by immunostaining using anti-MHC antibody after 6 d of myogenic differentiation. (J) Fusion index. (K) MHC protein abundance (mean ± SEM; $n = 8$ in each group, $^*P < 0.05$, $^{**}P < 0.01$). *PAX7* = paired box gene 7; MYOD = myogenic differentiation 1; MHC = myosin heavy chain.

a key role in the early stage of muscle differentiation but not in the late stage.

4. Discussion

It is widely believed that muscle fibers of domestic animals are formed before birth, and postnatal muscle growth is mainly achieved through the enlargement of existing muscle fibers (Albrecht et al., 2006; Du et al., 2010; Wegner et al., 2000). Satellite cells are precursors of skeletal muscle cells which differentiate and fuse into muscle fibers (Kadi et al., 2005), and the number and activity of satellite cells greatly affect the muscle growth rate of animals (Yin et al., 2013). The proliferation activity of satellite cells is most active in neonatal animals than in older animals (Carvajal Monroy et al., 2017; Velleman et al., 2014), thus nutritional interventions at the neonatal stage could change the number of satellite cells and affect later muscle growth to a greater extent.

Retinoic acid is an important regulator of muscle development. Retinoic acid enhances myogenic differentiation of human embryonic stem cells by increasing the number of myogenic

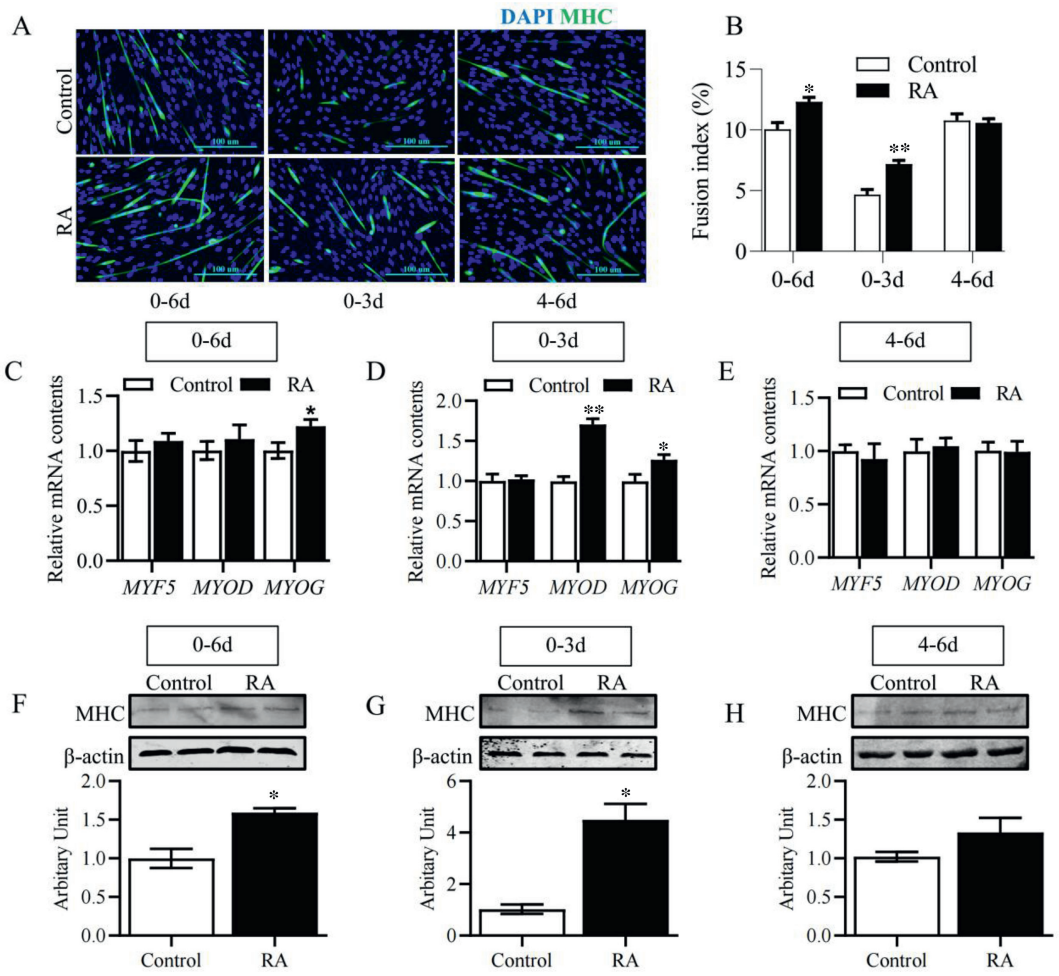

Fig. 4. Retinoic acid promoted the early differentiation of sheep primary myoblasts. (A) Immunofluorescence staining of myotubes using anti-MHC antibody at 3 stages. (B) Fusion index at 3 stages. (C-E) The level of myogenic mRNAs at 3 stages. (F–H) MHC protein abundance at 3 stages. (mean ± SEM; $n = 8$ in each group, $^{*}P < 0.05$, $^{**}P < 0.01$). RA = retinoic acid; *MYF5* = myogenic factor 5; *MYOD* = myogenic differentiation 1; *MYOG* = myogenin; DAPI = 4′,6-diamidino-2-phenylindole; MHC = myosin heavy chain.

progenitors (Ryan et al., 2012). In beef cattle, neonatal vitamin A injection increases the density of satellite cells and promotes muscle growth (Wang et al., 2018). Although the increase in immunofluorescence-labeled satellite cells was not statistically significant, neonatal vitamin A injection increased the mRNA abundance of the satellite cell marker, *PAX7*, a key transcription factor that is essential for regulating the expansion and differentiation of satellite cells (von Maltzahn et al., 2013), which is likely due to enhanced satellite cell proliferation and may have contributed to the improvement of muscle growth.

Retinoic acid also enhanced myogenic differentiation by activating RARs bound directly to mesoderm and skeletal muscle progenitor genes, such as *Wnt3a*, *Pax3*, and *Meox1,* activating β-catenin function and inhibiting bone morphogenetic protein (BMP) signaling (Halevy and Lerman, 1993; Kennedy et al., 2009). Retinoic acid upregulates the expression of myogenic regulatory factors (MRFs) *MYOD* (Reijntjes et al., 2010; Ryan et al., 2012), *MYF5* (Reijntjes et al., 2010), and *MYOG* (Kennedy et al., 2009) in myogenic cells of humans and different animal species. Consistently, we observed that neonatal vitamin A injection upregulated the expression of *MYOD* and *MYOG* in biopsied muscle tissue and increased the myogenic potential of mononuclear cells isolated from skeletal muscle of vitamin A-treated lambs.

Myogenic differentiation was a highly coordinated sequential program to generate mature skeletal muscle (Jang and Baik, 2013). Myoblasts differentiate into mononuclear myocytes at an early stage of differentiation and fuse into multinucleated myotubes at a later stage, which expresses many muscle structural proteins such as MHC, creatine kinase (MCK), and alpha-actin (Lluís et al., 2006). As a downstream component of IGF1 signaling, AKT and mTOR are the key regulators that promote skeletal muscle protein synthesis and prevent muscle protein degradation (Schiaffino and Mammucari, 2011). However, neonatal vitamin A injection did not affect Akt/mTOR signaling in lambs. We also observed no changes in the expression of MSTN and Smad3, proteins involved in major signaling that inhibits muscle growth (Allen and Unterman, 2007). When analyzed in vitro, RA promoted myogenesis of primary skeletal muscle-derived mononuclear cells only in the first 3 d of differentiation but not in the later stages. These data indicate that vitamin A and RA promote muscle growth mainly through increasing the number of myogenic progenitors and improving their myogenic potential in the early stage of muscle development but not by affecting muscle protein synthesis that occurs in the late stages.

Neonatal vitamin A injection is a promising nutritional strategy that economically and efficiently promotes muscle growth of

livestock animals. In beef cattle, 2 shots of 150,000 IU vitamin A at the neonatal stage increased body weight by 13.1% at weaning and by 8.8% at 308 d of age (Harris et al., 2018). In the current study, 4 shots of 7,500 IU vitamin A from birth to 3 wk of age caused a 17.5% increase in slaughter weight and a 16.8% increase in the loin eye area of lambs at 8 months of age. It is highly effective and easy to operate for livestock producers.

5. Conclusion

Neonatal intramuscular vitamin A injection upregulates *PAX7* expression and promotes the myogenic potential of satellite cells, which has a long term effective of promoting the muscle growth of lambs.

Author contributions

Pengkang Song: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing. **Xiaoyou Chen** and **Jiamin Zhao**: Investigation. **Qiang Li**, **Xinrui Li** and **Yu Wang**: Methodology. **Bo Wang**: Conceptualization, Data curation, Project administration, Supervision, Writing – review & editing. **Junxing Zhao:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. All the authors have read the paper and have agreed to be co-authors data Availability Statement.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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